T113
+Y12
2641

ATTEMPTED INDUCTION OF AUTO-IMMUNE PHENOMENA IN GUINEA PIGS BY MYXOVIRUS INFECTION

PHILIP WILLIAM ASKENASE

1965

MUDD
LIBRARY
Medical

YALE

MEDICAL LIBRARY

Digitized by the Internet Archive
in 2017 with funding from
Arcadia Fund

https://archive.org/details/attemptedinducti00aske

ATTEMPTED INDUCTION OF AUTO-IMMUNE PHENOMENA IN GUINEA PIGS BY MYXOVIRUS INFECTION

A Thesis presented to the faculty of the Yale University School of Medicine in partial fulfillment of the requirements of the degree of Doctor of Medicine

by

PHILIP WILLIAM ASKENASE, B.A.

Brown University 1961

The Department of Microbiology

Yale University School of Medicine

New Haven, Connecticut

1 May 1965

YALE MEDICAL
NOV 1965
LIBRARY
T113
Y12
2641

ACKNOWLEDGEMENTS

I wish especially to acknowledge the always enthusiastic assistance of Dr. E. Peter Isacson who graciously provided, in addition to his laboratory, sage advice and kind encouragement in the execution of these experiments.

In addition, I thank Drs. Ohtomo, Van Orden and Treffers for their advice on diffusion-in-gel techniques and Miss Lorraine Tate and Audrey Koch for their technical assistance. I also wish to thank Dr. Byron Waksman for kindly reading and evaluating this manuscript and Carole MacAnally for typing the final draft.

Finally, my thanks to the United States Public Health Service for their financial assistance and to the Staff of the Yale Computer Center for instruction in computer-sorter techniques which facilitated analysis of my data.

CONTENTS

PAGE

A. INTRODUCTION AND REVIEW OF THE LITERATURE

1. Viral mechanisms in the etiology of auto-immune phenomena 1
2. Modification of host antigenicity by direct viral action; *in vitro* demonstration 1
3. Modification of host antigenicity by direct viral action; *in vivo* demonstration 3
4. Viral utilization of host antigens..... 5
5. Antigenic modification of utilized host material..... 6
6. Modification of host antigens and immunological processes by viral influences on genetic mechanisms 7
7. Relevant clinical material..... 8

B. PLAN 9

C. MATERIALS AND METHODS

1. Hemagglutination test for viral specimens 10
2. Viral sources 10
3. Purification and concentration of viral material used to inoculate live guinea pigs 11
4. Purification and concentration of virus material used to inoculate suspensions of guinea pig organs 12

5. Animal care, inoculation and sera collections 13

6. Preparation of organ extracts 13

7. In vitro interaction of virus and guinea pig organs 14

8. Hemagglutination inhibition tests 14

9. Complement fixation tests..... 15

10. Precipitin tests 15

11. Immunoelectrophoresis..... 16

D. RESULTS

1. Hemagglutination tests 17

2. Hemagglutination inhibition tests..... 17

3. Diffusion-in-gel precipitin tests 18

4. Description of precipitation reactions..... 20

5. Relation of serum precipitins to anti-viral antibodies in guinea pigs experimentally exposed to the DA virus 22

6. Organ precipitins in commercial pooled guinea pig sera as a control 24

7. Organ precipitins present in serum drawn from guinea pigs before experimental exposure to live DA virus..... 25

8. Relation of organ precipitins to anti-viral antibodies according to mode of experimental exposure to live DA virus 26

9. Paired pre sera as controls 27

10. Results of data on changes induced in guinea pig organs by interaction with live DA virus 29

11. The character of the precipitation reaction 31

12. The character of the precipitating substances in organ extracts 32

13. Character of the serum precipitins 33

14. Results of Immunoelectrophoreses 34

15. Character and quantity of immune globulins contained in the organ extracts 35

E. DISCUSSION

1. The diffusion-in-gel technique as applied to the investigation of auto-immune phenomena 37

2. Relation between anti-viral antibodies and organ extract precipitins in inoculated guinea pigs 38

3. Relation between anti-viral antibodies and organ extract precipitins in infected guinea pigs 40

4. The nature of the organ extract precipitins 40

5. The nature of the precipitating substances in the organ extracts 43

F. SUMMARY 44

G. TABLES 1 to 13 45-57

H. FIGURES 1 to 3 ..... 57a, b, c

I. BIBLIOGRAPHY 58-66

A. INTRODUCTION AND REVIEW OF THE LITERATURE:

1. VIRAL MECHANISMS IN THE ETIOLOGY OF AUTO-IMMUNE PHENOMENA:

When immunological phenomena usually associated with resistance and allergy to exogenous substances seem to be directed against endogenous constituents of the organism producing them they are called auto-immune (1). Among the many etiological entities and pathogenic mechanisms that have been proposed to account for auto-immunity only those invoking viral induction will concern us here. The etiologies proposed include: modification of host antigens by direct viral action (2, 3, 4, 5, 6, 7, 8, 9, 10, 11); release of concealed host antigens by direct viral action (2, 12, 13); combination of viral and host antigens into new antigenic entities (14, 15); antigenic similarity between native viral and host substances (16); and finally, indirect modification of host antigens and immunological processes by viral influences on genetic mechanisms (17, 18, 19, 20).

2. MODIFICATION OF HOST ANTIGENICITY BY DIRECT VIRAL ACTION; "IN VITRO" DEMONSTRATION:

Receptor destroying enzyme (RDE) is a neuraminidase capable of splitting neuraminic acid from mucoprotein which may be present on cell surfaces. RDE is found in cultural filtrates from Vibrio cholera as well as on the surface of myxoviruses which include the various strains of influenza and para-influenza as well as the viruses of fowl plague, Newcastle disease and mumps (21). After myxoviruses agglutinate erythrocytes RDE causes elution with concomitant modification of receptors such that agglutination will not recur if fresh virus is added (22, 23).

This red cell alteration is accompanied by a 2% weight loss (24) and renders the cells agglutinable by "incomplete blocking" anti-Rh antibody if they are Rh positive and "panagglutinable" by a variety of human sera (24). Thus, there is some evidence for in vitro modification of antigenicity by myxoviruses.

In 1947 Burnet (11) noted that in the process of hemagglutination by influenza virus a new "T" antigen was released from human erythrocytes which on injection into rabbits produced anti-T antibodies capable of agglutinating influenza treated cells. At that time he stated that "the appearance of a new cellular antigen by the action of certain viruses or bacterial products on red cells raises the possibility that similar action in vivo might result in the production of auto-antibodies corresponding to the new antigen."

Just as sera prepared in animals immunized with the "T" antigen will agglutinate erythrocytes modified by myxoviruses it has long been recognized that immune sera from animals and patients infected with certain myxoviruses will agglutinate red cells modified by appropriate myxovirus incubation (22). In addition, even highly diluted sera from some patients with infectious mononucleosis or infectious hepatitis have been shown capable of agglutinating similarly modified red cells (25, 26, 27, 28).

Isacson (10), working with a newly characterized myxovirus called the DA virus, demonstrated that red cell alteration was due not only to the presence of viral antigenic material on the erythrocyte surface, but also from the direct action of viral RDE on the antigenicity of the surface itself. Red cells incubated with RDE alone were agglutinated to high titer by sera obtained from patients convelescing

from infectious hepatitis (10, 25). Pre-adsorbtion of these sera with DA virus failed to remove the agglutinins for RDE modified cells, whereas anti-viral HI titers were removed. When animals were immunized with inactivated virus their sera developed anti-viral agglutinating antibodies, but no agglutinins for RDE modified erythrocytes. Conversely, sera from animals immunized with RDE treated red cells contained agglutinating antibodies for RDE modified erythrocytes and no anti-viral agglutinating antibodies.

These experiments led Isacson to conclude that antibodies to altered host tissue arising subsequent to DA virus infection were not only due to persistence of viral antigen, but also from direct enzymatic modification of host cell antigenicity with subsequent auto-sensitization.

3. MODIFICATION OF HOST ANTIGENICITY BY DIRECT VIRAL ACTION; "IN VIVO" DEMONSTRATION:

The occurrence of in vivo hemagglutination, as has been noted by dissecting microscope aided inspection of the bulbar conjunctivae of animals infected with the hog cholera virus (8), raises the possibility of accompanying in vivo antigenic modification. Red cells removed from chicks infected with Newcastle disease virus (NDV) have been found to be modified in a way similar to NDV induced in vitro modification, and these altered red cells were found to persist beyond the time virus was detectable. The agglutinating antibodies in immune sera were shown to be directed against the modified cells and not perisiting virus or viral antigen because agglutinins remained while hemagglutination inhibition antibodies disappeared on pre-adsorbtion with NDV (5).

Related to the subject of in vivo alteration of cellular antigenicity by myxoviruses are the observations of Moolten (8, 9, 29) which have never been adequately confirmed. He found that red cells from patients viremic with NDV were agglutinated to high titer by sera from patients with NDV, mumps, and infectious mononucleosis (8) and that erythrocytes from a patient with infectious mononucleosis and another with systemic lupus erythematosis (SLE) were agglutinated by immune sera from cases of infectious mononucleosis and lymphocytic choriomeningitis respectively (9).

Moolten attempted to isolate viruses from the blood of patients with idiopathic hemolytic anemias and claimed success in 22 of 64 patients. Twelve of the 22 positives were listed as unidentified viruses, but were judged to be genuine isolates on the basis of lesions produced in inoculated chick embryos and hemagglutination by inoculated chorioallantoic membrane fluid. There was no mention of serial passage. On such "isolate" from a patient with Hodgkin's disease was used to immunize rabbits and the resulting sera was found capable of agglutinating washed red cells obtained from one of the patients with Hodgkin's disease. Another unidentified virus was twice isolated from a patient with SLE and was found capable of inducing the LE phenomena. Because of the multiplicity of in vitro modes of inducing the LE phenomena when using a known positive system it is unfortunate that no details of method used or of control results were given. Finally, eggs were inoculated with virus obtained from one of the SLE patients and volunteers who were injected with formalinized allantoic fluid developed sera that could inhibit the viral induced LE phenomena and plasma transfused from such a volunteer appeared to produce a temporary clinical remission (9).

Other investigators failed in vigorous attempts to isolate viruses in hemolytic anemias (30, 31, 32), but one of them (32), working with the electron microscope, claimed to recognize virus like particles attached to erythrocytes from patients with hemolytic anemia.

4. VIRAL UTILIZATION OF HOST ANTIGENS:

Another mode by which viruses, and in particular myxoviruses, might induce auto-immunity is by the combination of viral and host antigens into new antigens considered foreign by the host's immunological apparatus (14, 15) or by viral release of concealed endogenous antigens capable of auto-sensitization (2, 12, 13). Invaded cells appear to synthesize myxoviruses in separate parts and assemble the whole at the cellular membrane where the viral particle is released without rupture of the cell (33). Myxoviruses are unusual in their high lipid content and experimental evidence strongly suggests that much of this is host material incorporated in the process of viral synthesis and emergence from infected cells (34). Indeed, it has been demonstrated that the lipid composition of influenza virus reflects that of the host tissue it was grown in and that some viral lipid components were present in host cells before infection while other components were synthesized after infection through partial interference in the normal biosynthetic pathways of the host cell. These substances may be derived from host nuclear or cytoplasmic membrane as well as from cytoplasmic constituents (35).

Invoking Burnet's clonal selection theory of immunity (36) there are two modes by which structural utilization of host materials might enable myxoviruses to induce auto-immune phenomena. Endogenous antigens already well known to host immune processes as native constituents might combine with viral components

into new antigenic species interpreted as foreign by the host immune apparatus and thereby lead to auto-sensitization. The other possibility is that endogenous antigens concealed from the host immune apparatus at the time of establishment of immune tolerance are unmasked by viral action or are revealed to the immune apparatus by their incorporation into myxoviruses.

Mouse lung or chorioallantoic membrane antigens have been found to form an integral part of influenza viruses grown in these respective media (37, 86) and DA virus grown in tissue culture cells possessing blood group B activity emerges with B activity (38, 21) whereas ordinary methods fail to demonstrate B activity when DA is grown in tissues without inherent B activity.

5. ANTIGENIC MODIFICATION OF UTILIZED HOST MATERIAL:

Utilized host material may also be altered antigenically as is suggested by the electron micrographs of Morgan et al (39) which show influenza virus particles being enclosed in an outer envelope derived from host cytoplasmic membrane. Ferritin labeled antibodies from an animal immunized with the same influenza strain fixed to this outer coat of emerging viral particles, as well as to portions of the cytoplasmic membrane not involved in the budding process, whereas un-inoculated CAM cytoplasmic membranes failed to fix ferritin labeled antibody. This suggests that host material is incorporated into emerging myxoviruses and that this material and remaining host constituents may be antigenically changed in the process.

6. MODIFICATION OF HOST ANTIGENS AND IMMUNOLOGICAL PROCESSES BY VIRAL INFLUENCES ON GENETIC MECHANISMS:

The thought that host cellular antigenicity might be changed in the process of viral synthesis touches on the theory that the host may become immunologically altered by viral influences on genetic mechanisms. Again we find myxoviruses postulated as a prominent etiological factor in this possible mode of auto-sensitization for it has been stated that the myxovirus infected cell has a surface modified by the incorporation of a new molecular species into the cell membrane by a process normally operative in the cell (19). It is of related interest that herpes simplex virus has been shown capable of changing the antigenic structure of an infected line of replicating HEP-2 tissue culture cells (40) and that a new antigen is found among in vivo tissue cells transformed into a tumor by the polyoma virus. This antigen is unrelated to viral antigenicity and subsequent generations of daughter cells develop the new cellular antigen that is demonstrable in tumor producing cells after the virus is no longer detectable (41).

Hotchin (18) has found that the lymphocytic choriomeningitis virus in mice is capable of inducing acute or chronic auto-immune disease by the neonatal infection of an immunologically tolerant animal leading to a persisting tolerated infection. Under appropriate circumstances the latent virus may transform host cells by phenotypic mutation and sensitize host lymphocytes to viral antigens so that reactions become possible between the host immune system and the host cells. Hotchin has suggested that myxoviruses may phenotypically alter host cellular membrane to behave antigenically similar to the membrane enclosing the virus (20.

7. RELEVANT CLINICAL MATERIAL:

Clinical observations and investigations justify investigation of the role of viruses in auto-immune phenomena and disease. By use of complement-fixation techniques antibodies reacting with antigenic material derived from several organs have been demonstrated in acute and convalescent sera from patients with infectious hepatitis and infectious mononucleosis which are presumably viral diseases (42, 4, 16). Similar sera have also been shown to possess substances capable of reacting with various red cell antigens and with antigens used for serological test for syphilis (29). In addition, anti-thyroid antibodies have been demonstrated after mumps thyroiditis and anti-erythrocyte antibodies after viral pneumonia (16). Thrombocytopenia purpura following measles infection has been attributed to viral induced auto-sensitization (43) and the occurrence of thrombocytopenic purpura in the congenital rubella syndrome where virus has recently been demonstrated in placental and neo-natal tissues (44) raises the possibility of a similar disease mechanism.

B. PLAN

1. Grow, purify and concentrate some DA virus
2. Inoculate live guinea pigs with active virus
3. Inoculate suspensions of guinea pig organs with active virus
4. Allow live uninoculated guinea pigs to co-habitate with inoculated guinea pigs in the hope of resultant "natural" infection
5. Collect a series of pre and post sera from exposed guinea pigs
6. Prepare organ extracts from exposed animals and cell suspensions
7. Follow viral immunity and infection by hemagglutination inhibition titers in the sera of exposed guinea pigs
8. Attempt to demonstrate antibodies in the sera reactive with antigens in the organ extracts
9. Attempt to demonstrate any relation between anti-viral antibodies and anti-organ antibodies

C. MATERIALS AND METHODS

1. HEMAGGLUTINATION TEST FOR VIRAL SPECIMENS:

Fresh fowl venous blood was obtained from a Rhode Island red rooster using a heparinized syringe and centrifuged at 300g for 10 minutes. After plasma was removed the cells were resuspended in 0.85% saline and centrifuged once again. Two similar washings were followed by resuspension in saline so as to constitute 20% by volume for a stock source of fowl erythrocytes. Specimens for viral determination were made up in serial two-fold dilutions in saline, beginning at 1:10, in 0.5ml volumes. The stock erythrocytes were further diluted to 1.0% in saline and added, in 0.5ml volumes, to the two-fold dilutions. Tubes were shaken and the contents allowed to settle at room temperature for 45 minutes. Agglutinated red cells settle in clumps so as to diffusely fill the entire curvature of the tube's bottom rather than settling separately so as to form a dense button filling only the curvature's apex. Viral titer was read as the highest dilution giving agglutination of the final 0.5% red cell suspension.

2. VIRAL SOURCES:

The DA strain of parainfluenza type 5 virus was first isolated in a post-mortem specimen of whole human blood from a fatal case of presumed viral hepatitis (45, 46). It was grouped as a myxovirus and found to be biologically similar to the mumps-Newcastle disease virus subgroup (47), and finally antigenically classified as one of the three strains of parainfluenza type 5 virus (48).

Roux and three-ounce prescription bottles of monolayer rhesus monkey kidney culture were prepared by the Department of Epidemiology and Public Health according to well known methods (49, 50, 51, 52) and their growth medium (containing Hanks salt solution, 0.5% lactalbumin hydrolysate, 2% calf serum, phenol red and antibiotics) replaced with maintenance medium (identical except for Earles salt solution). Tissue culture fluid known to contain DA virus was similarly obtained and added in 1.0ml and 0.1ml volumes to the large and small bottles respectively. All transfers were done using sterile technique in a room receiving ultra-violet irradiation nightly. Cultures were incubated at 37° C. for one week and aseptically harvested by carefully pouring supernatant fluid off of cellular material into sterile flasks stored at -4° C. for later purification and concentration. Growth medium, maintenance medium, viral inoculum and harvest were tested for hemagglutination to follow the course of viral growth.

3. REMOVAL OF TISSUE CULTURE CONTAMINANTS (PURIFICATION) AND CONCENTRATION OF VIRAL MATERIAL USED TO INOCULATE LIVE GUINEA PIGS:

Harvest was thawed and centrifuged at 2000g for 30 minutes at 4° C. to remove tissue culture debris and 250ml of supernatant fluid centrifuged at 65,900g for 60 minutes at 4° C. to remove contaminants lighter than virus. Resulting pellets were resuspended to a volume of 25ml in sterilized Trizma buffered (pH 6.8 at 4° C.) normal saline, mixed with an equal volume of washed 20% guinea pig erythrocytes and shaken frequently at 4° C. for 45 minutes.

After centrifugation at 2000g for 15 minutes at 4° C., the packed agglutinated erythrocytes were resuspended in saline and shaken frequently for 45 minutes in a 37° C. waterbath to allow for viral elution. Centrifugation at 2000g for 15 minutes at 4° C. was repeated and the supernatant fluid centrifuged at 65,900g for 60 minutes at 4° C. Resulting pellets were brought to a volume of 25ml in saline and this concentrated and more fully purified viral source material was stored at -4° C. after hemagglutination testing along with supernatant fluids from centrifugation of the agglutinated red cells and from the ultra-centrifugation of the fluid eluted from these cells in order to follow virus through the processing.

4. PURIFICATION AND CONCENTRATION OF VIRUS MATERIAL USED TO INOCULATE "IN VITRO" SUSPENSIONS OF GUINEA PIG ORGANS:

Virus was grown in monolayer rhesus and green monkey kidney tissue cultures and 250ml of harvested fluid clarified by centrifugation and ultra-centrifugation as before. The resuspended pellets were admixed with 12.5% $BaSO_4$, shaken in the cold to adhere virus to $BaSO_4$ particles (53, 54), centrifuged at 2000g for 15 minutes at 4° C., and the supernatant fluid mixed with additional $BaSO_4$. Both resulting $BaSO_4$ pellets were washed twice in distilled water, virus was eluted in multiple washings with 0.25M sodium citrate buffer at 37° C. and these were tested for hemagglutination and then combined for ultra-centrifugation at 65,900g for 60 minutes at 4° C. Resulting pellets were resuspended in phosphate buffered saline (pH 7.4 at 37° C.) to a volume of 25 ml. and stored at -4°C. after hemagglutination testing to follow the course of viral processing.

5. ANIMAL CARE, INOCULATION AND SERA COLLECTIONS:

Adult and two week old baby guinea pigs were kept in separate cages and fed a standard laboratory diet. While the animals were under light ether anesthesia, blood was withdrawn by closed cardiac puncture and allowed to clot in a glass test tube. Sera was withdrawn after centrifugation and frozen for subsequent immunological testing.

Sterile virus containing isotonic fluid previously purified by guinea pig erythrocyte adsorbtion and concentrated by ultra-centrifugation was used for inoculation. Several of the adult and baby guinea pigs received 1.0ml intra-nasally and 2.0ml by the intra-peritoneal route, several others also received intra-dermal and intra-muscular inoculation in 0.5ml amounts, and several other animals received no virus, being placed in cages with inoculated animals in the hope that they would contract natural infections.

6. PREPARATION OF ORGAN EXTRACTS:

Guinea pigs were exsanguinated under light ether anesthesia and organs quickly removed and washed free of surface blood and debris in cooled saline. After mincing with a cooled scissors in a cooled glass tube they were mixed with 5 times their weight of cooled phosphate buffered saline (pH 7.4) (10 times for small organs such as gall bladder, trachea and thyroid) and homogenized for 10 minutes in a cooled teflon homogenizer and then briefly ultra-sonicated. The resulting materials were centrifuged at 550g for 15 minutes at 4° C. and supernatant fluids used as organ extracts. Protein content was determined by biuret colorometric methods.

Phenol-water extraction according to the method of Broberger and Perlmann (55) was performed on the liver of one guinea pig to obtain lipopolysaccharide antigens.

7. "IN VITRO" INTERACTION OF VIRUS AND GUINEA PIG ORGANS:

Organs were aseptically removed from an adult guinea pig under ether anesthesia and washed free of surface blood and debris in cooled sterile saline. They were then rubbed through a cooled U.S. standard sieve #50 (0.297mm) and the resulting cells and cell fragments suspended in phosphate buffered saline. Equal volumes of virus in phosphate buffered saline were added and the cells and virus allowed to react for 3 hours in a constantly shaking 37° waterbath. After resulting mixtures were centrifuged at 65,900g for 60 minutes at 4° C., the supernatant fluids were tested for hemagglutination and then frozen for later use as in vitro altered antigens in precipitin tests.

8. HEMAGGLUTINATION INHIBITION (HI) TESTING:

Immunization and infection of guinea pigs was followed by the ability of immune serum to inhibit the agglutination of erythrocytes by a standard dose of virus (52). Fluid containing virus was prepared in two-fold serial dilutions (beginning at 1:10) in phosphate buffered saline in 0.4ml amounts and mixed with 0.4ml of a 1% suspension of fowl erythrocytes. After 1 hour of settling, virus titer was read as the highest dilution capable of causing agglutination. This represented one hemagglutination (HA) unit and the dilution which would contain 4 HA units in 0.2ml was calculated. Serum was treated with trypsin and periodate to remove nonspecific inhibitors and/or nonspecific hemagglutinins and then prepared in serial twofold dilutions, beginning at 1:10, in 0.2ml amounts. Virus was added to each serum dilution and the HI titer read as the

highest dilution of serum inhibiting hemagglutination by 4 HA units of virus after 40 minutes.

9. COMPLEMENT FIXATION TESTS:

Tests were done according to a standard method (51) in serum albumin veronal buffer (pH 7.5) with calcium and magnesium added. Tubes containing 0.2ml volumes of diluted tissue antigen shown to have no anti-complementary activity when diluted 1:4, serum diluted 1:8 and 5 H_{50} units of complement were allowed to react overnight at 4° C. After adding 0.5ml of 1% sensitized sheep cells and shaking at 10 minute intervals in a 37° C. waterbath for 30 minutes, the tubes were centrifuged and hemolysis compared with standards prepared in distilled water.

10. PRECIPITIN TESTS:

Diffusion-in-gel methods according to Ouchterlony (56) and Crowle (57) were employed using borate buffered (pH 8.6 at 20° C.) 1.5% agar-agar spread on ordinary precleaned microscope slides. In four separate systems on each slide a 17 gauge agar cutting needle was used to drill 6 cylindrical peripheral wells set 5mm. from a single central well drilled with a 12 gauge needle. Organ extracts were placed in the peripheral wells using plain microhematocrit capillary tubes and serum was placed in the central wells using melting point capillary tubes. All reactants were thoroughly mixed using a Vortex Jr. Mixer before capillary tubes were filled. Wells were emptied, using empty capillary tubes, when bubbles were trapped or the well overflowed. No vigorous attempt was made to assure that all wells were of equal depth, but it was felt that they were all roughly the same volume since approximately 3ml of agar was allowed to distribute on each slide.

After wells were charged, the slides were laid on water saturated sponges and stored in closed plastic containers at constant room temperature. They were read using an indirect light source and a hand magnifying glass on each of several days following charging and finally stored at 4° C. for later review and photography.

11. IMMUNOELECTROPHORESIS:

Studies were done according to methods described by Crowle (57) on large microscope slides. Preliminary electrophoretic separations were performed in borate buffer (pH 8.6 and ionicity 0.05 at 20° C.) at 20 ma. for 1.5 hours.

D. RESULTS:

1. RESULTS OF HEMAGGLUTINATION (HA) TESTS:

Growth of virus, as measured by hemagglutination, took place after originally virus free tissue culture medium was inoculated with a small sample of fluid with HA titer of 1:256. Fluid harvested for *in vivo* interaction with guinea pig organs was purified and concentrated by erythrocyte adsorbtion and elution along with appropriate centrifugations, yielding grossly clarified fluid of one tenth the volume (250ml to 25ml) and twice the hemagglutination titer of the original (1:320 to 1:640). Fluid harvested for *in vitro* interaction with guinea pig organs was similarly processed by multiple $BaSO_4$ adsorptions and citrate elutions, yielding again grossly clarified fluid of one tenth the volume (250ml to 25ml), but with this technique hemagglutination titer quadrupled (1:320 to 1:1280). Thus, the live animals were inoculated with a 1:640 virus titer and the organ cell suspensions were inoculated with a 1:1280 virus titer.

2. RESULTS OF HEMAGGLUTINATION INHIBITION (HI) TESTS: (table 1)

Of the guinea pigs exposed to the DA virus and yielding serial sera by multiple cardiac punctures, 75% (9 of 12) developed a significant rise in HI titer (greater than four-fold). Among those with and without significant HI rises were guinea pigs who were directly inoculated as well as some who co-habitated with inoculated animals. Thus, mode of exposure played no part in reactivity to viral antigen. In the inoculated animals significant HI rises could have been due to infection or merely immunization, since they received live virus by intra-nasal and intra-peritoneal routes, but in the co-habitating guinea pigs with significant HI rises infection must

be assumed. Both adult and baby animals were able to develop significant antibody titers within five days after exposure, but the adults developed the highest titers. This may indicate previous exposure to the DA virus or an antigenically similar agent, or it might be a reflection of the greater immunological maturity of the adults who all developed significant rises in HI titer, while all of those without such rises were babies. Note that where pre sera were drawn on more than one occasion (guinea pigs #7, 10 and 12) there were no significant changes in HI titer before experimental inoculation and infection took place. Thus, there was no evidence of inadvertent infection previous to experimental exposure.

3. RESULTS OF DIFFUSION-IN-GEL PRECIPITIN TESTS:

Generally, the micro-Ouchterlony plates contained two to four sets of six peripheral wells around a single central well. Six different organ extracts from the same guinea pig were placed in similar positions in each peripheral hexagonal set and the remaining central wells were each charged with a separate serum from a serial set of pres and posts drawn from a single guinea pig. The sera were from the same animal that provided the organ extracts (autologous system) or from another guinea pig (isologous system). The sera or the organ extracts were from adult or baby animals, from inoculated or infected animals and from animals with or without significant HI rises. An additional set of organ extracts and suitable controls were exposed to virus in vitro. Close to 2500 separate interactions between sera and organ extracts were thus performed.

Precipitins were observed to occur in the interaction of serum and organ extract in many cases. These precipitations were analyzed to see if precipitins

bore any relation to viral exposure and attempts were made to understand the precipitin reaction itself and the nature of the substances involved in its production.

For a serial set of pre and post sera from a single exposed guinea pig, interacting with a single organ extract, the following types of responses were defined according to the observed occurrence of precipitin reactions:

1. When none of the pre sera precipitated with the extract and at least one post serum did, this was spoken of as a NEGATIVE PRE TO A POSITIVE POST.

2. When at least one of the pre sera precipitated with the extract and none of the post sera did, this was spoken of as a POSITIVE PRE TO A NEGATIVE POST.

3. When none of the pre sera and none of the post sera precipitated with the extract, this was spoken of as a NEGATIVE PRE TO A NEGATIVE POST.

4. When at least one of the pre sera and at least one of the post sera precipitated with the extract, this was spoken of as a POSITIVE PRE TO A POSITIVE POST.

For the pre sera of a serial set the following types of responses were defined according to the observed occurrence of precipitin reactions:

1. When there were two pres not precipitating with the extract, this was spoken of as PAIRED PRES BOTH NEGATIVE.

2. When the two pres both precipitated with the extract this was spoken of as PAIRED PRES BOTH POSITIVE.

3. When either pre precipitated with the extract and the other did not, this was spoken of as PAIRED PRES WHICH VARIED.

4. PAIRED PRES BOTH NEGATIVE and PAIRED PRES BOTH POSITIVE were therefore PAIRED PRES WITHOUT VARIANCE.

Thus, the interaction between a single set of serial sera and a single organ extract had to be either negative pre to positive post or positive pre to negative post or negative pre to negative post or positive pre to positive post and the paired pres had to be either both negative, both positive or with variance.

Tables 3-6 summarize the incidence of these possible kinds of interaction between serial serum groups and organ extracts from four different guinea pigs exposed to live DA virus, with the serum groups broken down according to HI titer against DA virus. These data are summarized for all four of the animals in table 7. Table 12 is a similar type of analysis of the interactions of organ extracts from a control guinea pig sacrificed before inoculation. Tables 9, 10 and 13 further divide up the interactions according to immuno-specificities and mode of viral exposure. Table 8 shows the results of interaction between all the serial serum sets and organ antigens exposed to live DA virus in vitro. Table 11 shows the variation pattern of the sera drawn from the guinea pigs before experimental exposure to DA virus.

4. DESCRIPTION OF PRECIPITATION REACTIONS:

The precipitation patterns appeared any time from a few hours after charging of the wells to a week later and they occured in the clear gel lying between the

hexagonally arranged peripheral wells (containing organ extracts from six different organs of the same guinea pig) and the single central well containing a serum sample (figure 1). Precipitations were atypical in character. Frequently seen was a ring of heavy precipitation running completely or incompletely around the area between the peripheral and central wells. It was invariably sharply demarcated from the normal clear gel on its central serum side and ran out diffusely in decreasing intensity of precipitation toward the peripheral organ extract side. In some instances the heavy inner margin of this precipitin band formed nearly a perfect circle, in others a hexagon with smooth corners, and in others ovaloid and assymetric patterns (some passing toward or through peripheral wells) were seen, but no matter what the shape of the band it connected with that formed by several neighboring serum-extract interactions. No spurs were noted.

In many instances other bands between individual serum-extract systems that did not connect up with similar reactions in adjacent serum-extract systems occurred in either the clear area on the central side of the interconnecting ring bank or on the peripheral side amid the diffuse precipitation. Since such multiplicity of bands in the interaction of a single serum and a single organ extract could be evidence of one or multiple reacting systems (57), any band pattern, whether single or multiple, was read as a positive precipitation reaction between serum and extract.

The interconnection of some of the precipitin bands into partial or complete rings or hexagons without spurs suggested that some of the substances in the separate organ extracts were identical in their specificity for serum precipitins (58).

If the serum precipitins were antibodies the extract substances were antigenically identical. The assymetrical interconnections of some bands could then have been evidence of "skewed identity" (57), occurring where there was appreciable disparity in concentration of identical substances in adjacent peripheral wells. If one of these adjacent identical antigens was of very low concentration the identical band would run toward and even into its well (59). The diffuseness of the identity band on the extract side might have been due to antigen excess (57).

It is known that for a single band the tips of the precipitation bend toward the lighter reactant and away from the heavier one (60). The isolated non-connecting bands occurring either side of the identity bands sometimes curved convex inward and sometimes convex outward or at times were relatively straight suggesting interactions of other reactants of different molecular weights and therefore possibly more than a single antigen antibody interaction.

5. RELATION OF SERUM PRECIPITINS TO ANTI-VIRAL ANTIBODIES IN GUINEA PIGS EXPERIMENTALLY EXPOSED TO THE DA VIRUS:

The sets of serial sera from guinea pigs exposed to live DA virus were divided into groups of sets with and without development of significant HI rise (greater than 4X). The incidence of the four types of serum changes from pre to post in precipitation with the organ extracts of guinea pig #2 were counted and expressed as percentages of the total number of responses to see if there was a relation between the development of precipitins and the development of anti-viral antibodies (table 3).

All of the serum conversions from negative pre to positive post appeared in serum sets with significant HI titer rise. None of the serum sets without similar rise in antibodies against components of the DA virus showed this development of precipitation factors for guinea pig organ extracts. Though many of the serum sets with significant rises in HI titer failed to develop these precipitins, 20% of them converted from negative pre to positive post [76% (55 of 72 responses) were negative pre to negative post vs. 20% (14 of 72 responses) were negative pre to positive post].

Similar analysis of serum set precipitins for extracts from other guinea pigs exposed to live DA virus in vivo failed to show as clear a relation between the development of precipitins for organ extracts and the development of HI antibodies, because conversions occurred from negative pre to positive post among sera sets without significant rises in HI titer.

For the organ extracts of guinea pig #34 the incidence of conversions from no precipitation with the pres to precipitation with the posts was the same in the sera sets developing anti-DA antibodies as in the sets without significant HI rise [10% (9 of 88 responses) vs. 9% (2 of 22 responses)][table 4]. However, against organ extracts from guinea pig #4 the sera sets with greater than 4X HI rise, when compared to the sera sets without such a rise, had three times the incidence of conversion from negative pre to positive post [35% (47 of 136 responses) vs. 12% (4 of 34 responses)][table 5]. Similarly, against organ extracts from guinea pig #8 the sera sets developing significant HI rises, had four times the conversion from no precipitins in the pres to precipitins in the posts [28% (33 of 120 responses) vs. 7% (2 of 30 responses)][table 6].

For all of these organ extracts from guinea pigs exposed to the DA virus in vivo (a total of 52 organs), all of the sera sets (10 sets), regardless of HI rise, had an overall frequency of 21% conversions from negative pre to positive post [111 of 520 (52 X 10) responses], but when the sera sets were broken into groups with and without significant rises in HI titer against DA virus, the sera developing greater than 4X HI titer rises in anti-viral antibody had three times greater development of organ precipitins [25% (103 of 416 responses) vs. 8% (8 of 104 responses)] [table 7].

6. ORGAN PRECIPITINS IN COMMERCIAL POOLED GUINEA PIG SERUM AS A CONTROL:

According to the data presented there was a suggestion that immunization or infection with the DA virus was associated with the development of serum substances capable of precipitating with extracts of organs from guinea pigs exposed to live DA virus. Unfortunately, animals kept separate from the inoculated animals to provide serial sera suitable for controls were lost to follow up. Commercial guinea pig complement, being just pooled guinea pig serum, was used as an indicator of the incidence of precipitins for guinea pig organ extracts exposed to live DA virus, among the general population of guinea pigs. This control serum had precipitins for 8% of the extracts from guinea pigs exposed to live DA virus in vivo. This was identical to the incidence of negative to positive conversions among serum sets without development of significant HI titer (table 7). The commercial pooled guinea pig sera had a HI titer against DA of 1:80.

Thus, pooled guinea pig sera had the same incidence of organ extract precipitins as the serum sets without significant HI rises, while sera sets developing anti-DA antibodies developed three times the incidence of organ extract precipitins. It was of course realized that although pooled commercial guinea pig sera could be regarded as a source of substances generally found in the serum of the guinea pig population at large, the number of animals whose sera was pooled and the exact nature of the processing of the sera was unknown. It was supposed that the commercial guinea pig sera were probably handled with extreme care so as not to disturb the quality and quantity of any inherent components, because of their intended use as a source of complement and of the well known instability of the components of complement.

7. ORGAN PRECIPITINS PRESENT IN SERUM DRAWN FROM GUINEA PIGS BEFORE EXPERIMENTAL EXPOSURE TO LIVE DA VIRUS:

I found it convenient to express the incidence of serial serum set's responses in percentages, but it was realized that the denominators involved were not identical, though they were felt to be of the same order of magnitude. Even if the mode of analysis of the data were accepted, continuation of the analysis along the same lines revealed a less clear relation between HI antibodies and diffusion-in-gel precipitins.

Many of the serial sets of sera contained precipitins in the serum samples drawn before experimental exposure of the contributing guinea pigs to live DA virus. Thus, against organ extracts from all the guinea pigs exposed to live DA virus *in vivo* the serum sets with greater than 4X HI rise had about the same incidence of conversion from positive pre to positive post as did the sera sets

without greater than 4X HI rise [18% (73 of 416 responses) vs. 21% (22 of 104 responses)][table 7]. Thus, all the sera sets regardless of HI rise had the same incidence of conversion from positive pre to positive post, and this was similar to the incidence of conversions from negative pre to positive post in all of the sera [18% (95 of 520 responses) vs. 21% (111 of 520 responses)][table 7]. If the incidence of positive pre to positive post were taken as the base line level of precipitins for organ extracts in the group of sera sets examined, the ones developing significant anti-viral antibody titers showed no increased incidence of precipitins, and there was no correlation between development of viral immunity and organ precipitins.

Strangely, there was among the data evidence supporting the converse. Namely, that the failure to develop a significant HI titer after exposure to live DA virus was associated with the obliteration of serum precipitins for organ extracts, because serum sets without greater than 4X HI rise had an eighteen times greater incidence of conversion from positive pre to negative post, when compared to sera sets with greater than 4X HI rise [9% (9 of 104 responses) vs. $<$0.5% (2 of 416 responses)][table 7].

8. RELATION OF ORGAN PRECIPITINS TO ANTI-VIRAL ANTIBODIES ACCORDING TO MODE OF EXPERIMENTAL EXPOSURE TO LIVE DA VIRUS:

When the serum sets from animals directly inoculated with live DA virus were examined for the incidence of organ extract precipitins, a dual association of extract precipitins and immune antibodies was noted. All conversions from negative pre to positive post occurred in the sera sets from inoculated animals developing significant rises in HI titer, while virtually all of the reverse

conversion from positive pre to negative post occurred in sera sets from inoculated animals failing to show significant rises in HI titer (table 9). Neither effect was noted in the serial sera from co-habitators where animals with significant HI rises had virtually the same frequency of negative pre to positive post conversions as those without such a rise [17% (34 of 207 responses) vs. 22% (15 of 69 responses)] and where positive pre to negative post conversions were negligible in both groups (table 10).

Thus, when an animal without serum precipitins for guinea pig organ extracts was inoculated with live DA virus he simultaneously developed such precipitins along with HI antibodies, while inoculation of animals already possessing precipitins resulted in elimination of the precipitins for guinea pig organ extracts and failure to develop anti-viral antibodies.

Finally, animals naturally exposed and presumably infected (greater than 4X HI rise) developed no rise in organ extract precipitins when compared to similarly exposed guinea pigs who did not become infected.

9. PAIRED PRE SERA AS CONTROLS:

Another sort of control was to observe the incidence of organ extract precipitins in sera obtained on two occasions from the same animal prior to experimental exposure to virus. No comparisons of possible paired pre responses between sera sets with greater than 4X HI rise and those without rises could be made, because all of the animals from whom paired pres were obtained developed significant HI rises. The paired pres in these sets were found to vary in their possession of organ precipitins with about the frequency that all of the sera sets

converted from negative to positive or from positive to positive [15% (16 of 104 responses) [table 11] vs. 21% (111 of 520 responses) [table 7] vs. 18% (95 of 520 responses) [table 7]]. Thus, if this were used as the control frequency of organ precipitins occurring in one instance and not in another in the sera of a single guinea pig, the incidence of conversions from negative to positive was no greater and precipitins did not follow HI antibodies.

However, when varying pre serum pairs were examined to see the direction of their variance it was found that in 90% the variance was from an initial negative to a latter positive, suggesting that the development of serum precipitins was related to the transfer of the animals from the farm to the laboratory or their lives from time of arrival up until experimental exposure to live DA virus. Note that 88% of this paired pre with variance group were also included in the large positive to positive group. Therefore, they were almost uniformly part of sera sets whose interaction with organ extracts went from an initial lack of precipitation; to a later precipitation in serum still drawn previous to experimental viral exposure; to precipitation in one and often all sera samples drawn after intentional viral exposure. Thus, the data concerning the appreciable incidence of paired pre variance and of conversion from positive pre to positive post, might at least be interpreted as favoring a relation between serum precipitins and events in the lives of the animals during their stay in the laboratory.

Perhaps they picked up an accidental laboratory infection with DA virus which might have been present on the persons working with the virus. If this were so then the development of precipitins might have been initiated by such accidental exposure and enforced by the later intentional exposure. However,

recall that when paired pres were tested for HI titer therewere no significant changes before experimental exposure took place. The possibility remained that the development of precipitins preceded the development of HI antibodies as is the case in many instances where different factors develop at different rates after exposure to an infectious agent. Note that all of the animals from whom paired pres were obtained, and thus all of those doubly handled, developed significant HI titers (table 11). The possibility also remained that the development of precipitins was related to other environmental exposures possible in transfer from one place to another or in the adoption of a different diet fed by different animal keepers.

10. RESULTS OF DATA ON CHANGES INDUCED IN GUINEA PIG ORGANS BY INTERACTION WITH LIVE DA VIRUS:

When the precipitin reactions occurring in the micro-Ouchterlony plates were analyzed to see if DA virus had induced the ability of organ extracts to precipitate with guinea pig serum factors, the same difficulty with good controls and clear results seen in the analysis of the relation between precipitins and anti-viral antibodies was encountered. Guinea pig #5 was sacrificed before inoculations began so that control organs not experimentally exposed to live virus would be available for comparison with organ extracts from inoculated and infected animals. These control organ extracts precipitated with serial sets of immune sera to a similar over all degree as did the extracts from guinea pigs experimentally exposed to DA virus (table 12). It thus seemed that the occurrence of precipitating substances in organ extracts had nothing to do with viral exposure. However, the analysis of serum precipitin responses suggested

that the guinea pigs were somehow modified during their stay in the laboratory and that this change might have been accidental exposure to DA virus. If this were so then the organs of guinea pig #5 were not suitable controls and may have been accidentally exposed to the same virus the others were intentionally exposed to.

There were insufficient data to draw any conclusion about the degree of organ change associated with different modes of in vivo viral exposure. Separation of the interacting serum sets and organ extracts into reactions between sera and organs from the same guinea pig and reactions between sera of one guinea pig and organs of another guinea pig showed twice the conversion from negative pre to positive post in the autologous systems compared to the isologous systems. On the other hand, the isologous systems had all of the positive pre to negative posts and eight times the positive pre to positive posts (table 13).

Extracts of guinea pig organs having interacted with virus in vitro, as well as controls incubated with saline, uniformly failed to show precipitation with any of the serial sera sets from guinea pigs exposed to DA virus in vivo (table 8). In view of the precipitations these sera showed with organ extracts from animals that interacted with virus in vivo (table 7), as well as with extracts from an animal without laboratory viral contact (table 12), the discrepancy was thought due to the difference in preparation of organ extracts exposed in vivo compared to those exposed in vitro. The former were freed from homogenation and ultra-sonication debris by centrifugation at 550g, while the latter were freed from sieving and ultra-sonication debris by ultra-centrifugation and therefore discarding of the sediment containing cellular fragments, particles

and macro-molecules possibly able to precipitate with sera in the Ouchterlony plates. This supposition could have been proven had samples of the control and experimental cell suspensions been handled in a similar manner as the organ extracts from animals interacting with the virus in vivo. Ultra-centrifugation was performed to free viral interacting host antigenic material from the added virus so that precipitins could be interpreted as reactions between sera and tissue and not between viral antigen and known immune sera. This maneuver was unnecessary because even guinea pig sera with fairly high HI titer (1:1280) failed to precipitate with fluid containing viral titers of the same order of magnitude used to inoculate the organ suspensions, nor could ether extracts of DA virus precipitate with the immune guinea pig sera in the precipitin system employed.

11. THE CHARACTER OF THE PRECIPITATION REACTION:

Running diffusion-in-gel precipitin plates in the cold (0-4° C.) should bring out more subtle bands and sharpen existing ones compared to running the plates at higher temperatures (57). My precipitin bands seen at room temperature were decreased in number and intensity when control plates were rerun at 4° C. and were sharper, of increased intensity and in greater numbers when the plates were run at 37° C. (naturally the plates in the cold were read appropriately later and those at 37° C. appropriately earlier than the 20° C. plates). This anomalous behavior and the impression that in some of the precipitation patterns peripheral organ extracts seemed to be influencing the reactivity of others in the same plate, suggested the possibility that some sort of enzymatic influence of one extract on another was being detected and that this was enhanced by more physiological temperatures.

A control plate with one of the most active of the organ antigens replaced by saline gave precipitation of less intensity at adjacent sites, but the overall ring pattern persisted and even ran through the area between the serum and the well filled with saline. Increasingly more extensive controls deleting supposedly interfering extracts, failed to substantiate the hypothesis that one extract was influencing the ability of another to precipitate with serum. Controls with just the peripheral wells filled with known active antigens and the central well filled with saline failed to show any precipitation as was the case when serum was allowed to run against saline in all the peripheral wells.

Changing concentrations, total quantities, or distances of reactants just changed the placement of the same bands and their time of development in a manner similar to known specific antigen-antibody systems (57). When the organ extracts were incubated alone at 56° C. for 30 minutes prior to placement in the wells, or the same was done with the sera, the bands appeared, and formed sharper symmetrical corners of identity compared to the rings they showed without such treatment. This enhancement with heating might have been from the elimination of non-specific inhibitors which is commonly employed in other immunological reactions such as the hemagglutination inhibition test used in these experiments (52).

12. CHARACTER OF THE PRECIPITATING SUBSTANCES IN ORGAN EXTRACTS:

Centrifugation of the organ extracts at 1000g for 30 minutes at 20° C., removal of the supernatant fluid, triple washing of the pellets in saline, and saline resuspension of the washed pellets to original volume was performed.

The initially removed supernatant fluids no longer precipitated with known active sera, while the triply washed and resuspended pellets gave the original precipitation patterns. Is it possible for an organic macro-molecule to be heavy enough to be easily sedimented and yet small enough to diffuse out through the molecular sieve of the agar gel which is known to limit the passage of large molecules (59)?

Mixing organ extracts with equal volumes of 0. 25% trypsin and incubation of separate aliquots at 37° C. for 5 min., 15 min., 1 hr., and 12 hours and then running these against sera in the Ouchterlony plates failed to inhibit the development of the usual precipitin bands. The precipitating organ extract substances, might not, therefore, have been proteins, although it is conceiveable that susceptible peptide bonds were protected or not present. In addition, macro-molecules other than proteins are known to be antigenic.

13. CHARACTER OF THE SERUM PRECIPITINS:

Precipitations could not have been between virus or viral components persisting in the organ extracts of animals exposed to live virus, because neither virus alone or ether extracts of DA virus precipitated with known immune sera possessing HI antibodies and precipitins. In addition, it was hard to conceive of virus sedimenting out at 1000g unless it were fixed to tissue fragments, but then it would not have been able to diffuse through the agar to precipitate with the serum. Did the viral antigen possibly in the organ extracts free itself from tissue fragments once in the well? This possibility, was eliminated by obtaining the same precipitating bands even when the sera were adsorbed with virus or ether extracts of virus before being placed in their wells.

Was it possible that the material used to inoculate the guinea pigs still contained minute, but immunologically active amounts of monkey kidney antigenic material and that the inoculated animals had developed anti-monkey-kidney antibodies capable of precipitating with cross reacting substances in guinea pig organ extracts? It was hard to conceive of the same mechanism at work in the naturally infected animals, and pre-incubation of sera with monkey kidney tissue culture fluid failed to remove precipitins for organ extracts.

Finally, it might be suggested that "T" antigen had been released when virus was agglutinated with guinea pig erythrocytes in the method used to purify and concentrate virus for inoculation. If the organ extracts then contained substances with antigenicity similar to that of "T" substance, and this material had been inoculated along with virus, the precipitins were then probably anti-"T" antibodies. However, it seemed unlikely that "T" substance would sediment with the virus on ultra-centrifugation or would be transmitted to co-habitating guinea pigs who also developed precipitins.

14. RESULTS OF IMMUNOELECTROPHORESES:

Immunoelectrophoretic analysis of sera was carried out to see if the precipitins migrated as immune globulins and to see if they were composed of more than one substance. After preliminary electrophoresis of serum a nearby trough well filled with organ extract and the two allowed to diffuse towards each other. Commercial anti-guinea-pig-globulin made in rabbits was placed in a trough near pooled guinea pig serum that had been electrophoresed to serve as control.

The apex of the single precipitin band between serum and extract showed the serum precipitin's sum total of migration had been nil or slightly towards the positive (figure 2). Precipitins therefore appeared to migrate as either $alpha_2$ or $beta_1$ globulins which constitute many of the binding and carrying proteins of serum such as haptoglobin, ceruloplasmin, thyroxin-binding-globulin and transferrin (57). Similar analysis using antisera made against specific globulin fractions might have shown whether there were any $beta_{2A}$ or $beta_{2M}$ globulins which also migrate in this area. $Beta_{2M}$ globulins are known to be the initial immune globulins produced by an immunized animal (61).

No precipitins were obtained when preliminary electrophoresis of organ extracts was attempted.

15. CHARACTER AND QUANTITY OF IMMUNE GLOBULINS CONTAINED IN THE ORGAN EXTRACTS:

An attempt was made to analyze the character and quantity of immune globulins in the organ extracts that came from guinea pigs exposed to DA virus *in vivo*. Though the organs had not been perfused and undoubtedly retained lymphocytic elements bearing immune globulins, they had been washed free of surface and draining blood and diluted 1:5 before homogenization. It was known that there exists a linear relation between the log concentration of a reactant and the distance of the corresponding precipitation band from a fixed index line such as the interface between the depot of the reactant and the gel layer (59). Therefore, in a known immunospecific system with 11mm. between antigen and antibody wells, a doubling or halving of the concentration of either will shift the precipitation line by a millimeter in an appropriate direction (62).

This fact was confirmed in my agar plates using anti-guinea pig globulin and pooled guinea pig sera as antibody and antigen respectively, and although more precise quantitation of immunodiffusion precipitations were available (63, 64) this was deemed sufficient for my purposes.

The organ extracts were found to contain immune globulins, but in no greater over all quantity than would be expected by virtue of contained blood and lymphocytic elements. Thus, it could not be said that there was an increased quantity of immune globulins fixed to the organ extracts according to this simple Coons-like modification of the diffusion-in-gel technique.

However, when the organ extracts were placed in the wells of the immunoelectrophoresis plates and underwent preliminary electrophoresis before being allowed to diffuse toward troughs charged with anti-guinea-pig-globulin, it seemed that the predominant precipitating substances in many tissue extracts behaved like alpha or beta globulins migrating almost exactly as the precipitins in immune guinea pig sera had (figure 3). Thus, perhaps the precipitins demonstrated in the serial sera of guinea pigs exposed to live DA virus were attracted to the organs in vivo.

E. DISCUSSION:

1. THE DIFFUSION-IN-GEL TECHNIQUE AS APPLIED TO THE INVESTIGATION OF AUTO-IMMUNE PHENOMENA:

At present the three experimental tools most useful in the demonstration of reactions between antibodies and organ antigens are the auto-immune complement fixation (AICF) test; the tanned red cell technique of Boyden; and diffusion-in-gel techniques (65). There exists a broad experience with diffusion-in-gel demonstration of anti-organ antibodies (55, 66, 67, 68, 69) and Crowle claims that such "analyses seem to be outstandingly useful for detecting and especially identifying antibodies produced by an animal which react with his own tissues" (57). On the other hand, the AICF test has proved useful not only in demonstrating the existence of auto-immune phenomena, but in characterizing the organ antigens and serum antibodies involved (4, 70, 71), and various studies, especially the extensive experimental work on auto-immune thyroiditis, have demonstrated that precipitin techniques are the least sensitive of the three (72, 68, 73, 70, 74). Conversely, there are isolated reports of precipitin tests being positive where AICF tests were not (75). It was of course understood that the diffusion-in-gel technique is a precipitation test and thus a method of detecting precipitating substances that might be antigens and antibodies or less specific macro-molecular precipitators (76).

The small volume and great number of separate sera and organ extracts to be examined in these experiments, meant that many reactions, probably exhaustive of materials, would be required to maximally survey the possible antigen-antibody reactions. The precipitin technique was chosen for this study because inherent

simplicity and double diffusion micro-techniques enabled me to run nearly 2500 separate immunological tests, before exhausting initially modest serum and extract samples. It was hoped that this preliminary survey would indicate areas where more refined techniques could be applied in further elucidating the possible induction of auto-immunity by myxoviruses.

2. RELATION BETWEEN ANTI-VIRAL ANTIBODIES AND ORGAN EXTRACT PRECIPITINS IN INOCULATED GUINEA PIGS:

Compared to the level of organ extract precipitins found in commercial pooled guinea pig serum the inoculated animals that developed significant anti-viral immunity seemed to concommitantly develop precipitins for guinea pig organ extracts, but just as often animals had precipitins both before and after inoculation and had paired pre control sera that varied in precipitin content. Thus, using the serum of other guinea pigs prepared in an unknown fashion as a control, the development of organ extract precipitins and anti-viral antibodies seemed related, while using the serum of the guinea pigs who were inoculated as their own controls, no relation was borne out. The latter "positive to positive" and "paired pres with variance" control groups of serum sets were virtually all from the same animals who apparently entered the laboratory without precipitins and then began to develop them before experimental inoculation. The fact that the guinea pigs doubly handled before inoculation invariably developed HI titers supported the suggestion that accidental exposure to DA had occurred before inoculation and that anti-organ precipitins developed before HI antibodies. However, from the data at hand no definite relation between anti-viral antibodies and organ extract precipitins was demonstrated.

The disappearance of pre-existing precipitins in animals who failed to develop significant HI titers following live virus inoculation and their development in those responding with significant titers was remarkable. It suggested that susceptibility to immunization and possibly infection with DA virus was accompanied by failure to possess organ extract precipitins and that protection from immunization and possibly infection was associated with possession of extract precipitins that disappeared subsequent to inoculation, as if they were consumed.

Perhaps some prior modifying organ insult both allowed the development of auto-precipitins and rendered organs insusceptible to DA infection, as erythrocytes agglutinated with myxovirus will not agglutinate again to fresh virus after elution. If such organ changes were phenotypic, then physiological cellular replacement and serum protein catabolism would result in the original situation of no precipitins plus susceptible organs able to accept DA infection and possible antigenic modification with resulting precipitin development. Certainly repetition of these experiments with re-inoculation of guinea pigs failing to develop significant HI titers and losing precipitins would aid investigation of these speculations.

The presence of a serum factor whose absence rendered guinea pigs susceptible to immunization and perhaps infection with live DA virus, and whose presence protected the animals from immunization and perhaps infection seemed analogous to the interferon system where viral infection leads to a host substance protecting other cells from viral infection (77). Much experimentation would be necessary before further speculation could be made on the relation of the protective precipitins demonstrated herein and the complicated interferon system.

3. RELATION BETWEEN ANTI-VIRAL ANTIBODIES AND ORGAN EXTRACT PRECIPITINS IN INFECTED GUINEA PIGS:

Comparison of tables 9 and 10 indicates that failure to respond to viral exposure in animals possessing organ precipitins and response in those without precipitins did not occur in the co-habitating group who had negligible pre-existing organ extract precipitins and thus should have all been susceptible to viral infection according to the hypotheses advanced in the previous section. The failure of some co-habitants to develop significant HI titers might mean that if organ extract precipitins were protective their action was restricted to immunization and not infection. Of course, all of the guinea pigs possessing organ extract precipitins might have been fortuitously chosen for inoculation and the failure of some co-habitants to become infected may have been a reflection of the usual "natural" take rate of DA infection given variations in inoculum dosage and host factors.

Serum sets from this more naturally exposed group developed organ extract precipitins as frequently in the infected as in the uninfected group. Such a clearly negative result in a group of guinea pigs whose viral exposure was probably not compromised by tissue culture contaminants, immunization trauma or unnatural route of entry, represented the best evidence in the data under consideration that anti-DA antibodies and organ extract precipitins were unrelated.

4. THE NATURE OF THE ORGAN EXTRACT PRECIPITINS:

The nature of the precipitins and precipitating substances was only superficially examined due to insufficient remaining materials or time.

The immunoelectrophoretic analyses suggested that the serum precipitins had electrophoretic mobility properties in common with the serum fraction known to contain globulins with specific and non-specific affinities for various substances, (such as transferrin, thyroxin-binding-globulin and haptoglobulin) as well as globulin antibodies manufactured early in the response to an antigenic stimulus. Immunoelectrophoreses also demonstrated substances with electrophoretic mobility similar to these serum factors were fixed to the organ extracts. It was thus suggested that serum factors, which might be carrying or binding globulins or antibodies, were able to react with organ extract factors in vitro and were fixed to the organs in vivo.

In the analysis of the relation between these serum precipitating substances for guinea pig organ extracts and anti-viral antibodies, it appeared that when absent the precipitins rendered guinea pigs susceptible to immunization and perhaps infection with live DA virus and when present protected the animals from immunization and perhaps infection. Noteworthy in this regard is the fact that transferrin, a serum $beta_1$ globulin with immunoelectrophoretic mobility similar to that of my serum precipitins, has been shown capable of inhibiting viral multiplication (87). Further chemical, immunological and biological experiments seem indicated to explore this possible relation between transferrin and the protective serum precipitin for guinea pig organ extracts demonstrated herein.

Complement fixation tests were carried out with the serial sera and organ extracts of a single animal with uniform failure to demonstrate auto-

antibodies (table 2). The fact that some of these same extract-serum interactions gave the typical precipitations referred to in this paper argues against the precipitations representing interaction between antigen and antibody, though this may have been another exception to the general finding that the AICF test is more sensitive than the precipitin test in the detection of antibody and tissue antigen reaction.

Even if the complement fixation results were dismissed, rigorous definition did not allow the serum precipitins to be called antibodies in that they were only demonstrated to possess some of the in vitro properties possessed by antibodies (78). Major criteria such as demonstration of their production subsequent to antigenic exposure; appearance after a well defined induction period; early intensified response following anamnestic stimuli; and reaction only with specific antigen or closely allied substance were not adequately demonstrated. Further purification of the sedimentable and washable precipitating substances in the organ extracts followed by immunization of guinea pigs with this material might be a future course in attacking these problems.

It was realized that even if these precipitins were satisfactorily proven antibodies, the question of their significance would still be unanswered, for it is well established that the mere presence of auto-antibodies does not necessarily cause pathology (78, 12), although such humoral substances could act in synergy with sensitized lymphocytes (12) or circulating complement (2, 16) to cause autopathology. It is also possible that circulating auto-antibodies arise secondary to tissue alteration through already well recognized pathogenic mechanisms (12) or that it is physiological for animals to possess serum factors capable of acting like

antibodies in their in vitro reactions with tissue antigens (71, 79, 80, 81, 82, 83).

5. THE NATURE OF THE PRECIPITATING SUBSTANCES IN THE ORGAN EXTRACTS:

The nature of the precipitating substances in the organ extracts remained indeterminate and the riddle of how these undoubted organic macro-molecules were large enough to be sedimented at speeds usually suitable for settling of cellular elements and yet small enough to diffuse through agar gel (known not to allow the passage of large molecules) remained unsolved. More time and materials might have allowed rigorous characterization of the physical, chemical and biological character of these peculiarly behaving substances.

The fact that extracts from a guinea pig not experimentally exposed to virus showed equal precipitation ability compared to extracts from exposed animals, argued against the ability of DA virus to modify host antigenicity, but the possibility of accidental events prior to intentional exposure compromised this negative result.

The in vitro attempt to alter antigens was totally unsuccessful due largely to the discarding of potentially precipitating substances in a vigorous effort to exclude viral antigens. This portion of these experiments warrants redoing with the knowledge that virus will not precipitate with immune sera if titer of both are not extremely high, but such low titered immune sera can still react with organ extracts. The inability to demonstrate precipitation between known immune sera and DA virus or ether extracts of DA virus confirms the experience of others (84, 85, 47) that high titered virus and serum are necessary. Precipitation has been demonstrated between influenza virus with HA titer of 1:10,000 and serum with HI titer of 1:16,000, whereas maximum HA and HI titer reached here was 1:1280.

These experiments failed to confirm the findings of Broberger and Perlman (55) that extraction of reactive antigens was enhanced by phenol in water techniques.

F. SUMMARY:

1. A group of guinea pigs was inoculated and infected with the DA type of myxovirus and their serial sera analyzed for anti-viral antibodies by hemagglutination inhibition tests and for precipitins against guinea pig organ extracts by micro-Ouchterlony diffusion-in-gel techniques.
2. Seventy-five per cent of the exposed guinea pigs developed significant hemagglutination inhibition titers and anti-organ precipitins were demonstrated in many of the serial sera.
3. The anti-viral antibody titers and organ extract precipitins were compared and no definite relation between the development of anti-viral antibodies and organ extract precipitins was demonstrated.
4. Guinea pig organ extract precipitins arose in the sera of some guinea pigs subsequent to transfer from the farm to the laboratory, but before experimental exposure to virus.
5. These spontaneous organ extract precipitins were absent in those inoculated guinea pigs developing significant rises in hemagglutination inhibition titers and present in those not showing such rises.
6. Preliminary characterization of serum and organ factors participating in precipitation reactions was attempted. There was some suggestion that the demonstrated serum precipitins for organ extracts were related to the family of binding or immune globulins and that these factors might have been fixed to the organs *in vivo*.

Table 1

SUMMARY OF HEMAGGLUTINATION INHIBITION (HI) TESTS:

guinea pig #	guinea pig age	mode of exposure	day sera obtained relative to exposure	HI titer
1	adult	inoculated	-13	1:10
			+ 5	1:40
			+14	1:1280
4	adult	infected	-13	1:10
			+ 5	1:320
			+14	1:160
6	baby	inoculated	-13	1:10
			+ 5	1:80
7	baby	infected	-13	1:10
			- 7	1:10
			+ 5	1:40
			+14	1:40
8	baby	inoculated	-13	1:10
			+ 5	1:40
			+ 6	1:20
10	baby	infected	-12	1:10
			- 7	1:10
			+ 5	1:80
11	baby	infected	-13	1:10
			+ 5	1:80
12	baby	inoculated	-13	1:10
			- 7	1:20
			+ 5	1:80
13	baby	inoculated	- 7	1:10
			+ 5	1:20
14	baby	inoculated	- 7	1:20
			+ 5	1:20
16	baby	infected	- 7	1:10
			+ 5	1:20
34	adult	inoculated	- 8	1:10
			+ 6	1:160

Table 2

SUMMARY OF COMPLEMENT FIXATION TESTS:

organ antigens from guinea pig #8	sera from guinea pig #8 pre 7/10	pre 7/25	post 7/30	post 7/31	antigen alone	antigen + complement
adrenal	1	1	1	1	4	1
brain	-	1	2	1	4	1
colon	-	1	2	1	4	1
esophagus	0	0	1	1	4	1
gall bladder	0	0	0	0	4	0
heart	-	-	1	1	4	1
jejenum	-	2	2	1	4	2
ilium I	-	-	1	1	4	1
ilium II	-	2	2	1	4	1
kidney	-	1	2	1	4	1
liver	1	1	1	1	4	1
lung	1	1	1	1	4	1
ovary	-	0	1	0	4	1
spleen	-	-	1	1	4	1
stomach	1	1	1	1	3	1
striated muscle	-	0	1	1	4	0
trachea	0	0	1	1	4	1
tubes	-	0	1	0	4	1
sera alone	-	4	4	4	-	-
sera + complement	0	0	0	0	-	-

Note: "-" denotes no test run; "0" denotes 100% lysis or no complement fixation; "4" denotes no lysis or 100% complement fixation (except in the antigen alone and sera alone controls where there was no complement and therefore "4" denotes no hemolysis or no complementary or hemolytic activity in the sera or antigens themselves)

Table 3

SUMMARY OF DIFFUSION-IN-GEL PRECIPITIN TESTS BETWEEN GROUPS OF SERIAL GUINEA PIG SERA AND VARIOUS ORGAN EXTRACTS OF ADULT GUINEA PIG #2 WHO WAS SACRIFICED ONE HOUR AFTER INTRA-NASAL AND INTRA-PERITONEAL INOCULATION OF LIVE DA VIRUS:

serum changes observed	serum sets with greater than 4X HI rise		serum sets without greater than 4X HI rise	
	#	%	#	%
negative pre to positive post	14	20	0	0
positive pre to negative post	0	0	1	5
negative pre to negative post	55	76	17	95
positive pre to positive post	3	4	0	0
Total	72	100	18	100

Table 4

SUMMARY OF DIFFUSION-IN-GEL PRECIPITIN TESTS BETWEEN GROUPS OF SERIAL GUINEA PIG SERA AND VARIOUS ORGAN EXTRACTS OF ADULT GUINEA PIG #34 WHO WAS SACRIFICED TWO WEEKS AFTER INTRA-NASAL, INTRA-PERITONEAL, INTRA-MUSCULAR AND INTRA-DERMAL INOCULATION OF LIVE DA VIRUS. GUINEA PIG #34 DEVELOPED A SIGNIFICANT HI TITER.

serum changes observed	serum sets with greater than 4X HI rise		serum sets without greater than 4X HI rise	
	#	%	#	%
negative pre to positive post	9	10	2	9
positive pre to negative post	0	0	0	0
negative pre to negative post	79	90	20	91
positive pre to positive post	0	0	0	0
Total	88	100	22	100

Table 5

SUMMARY OF DIFFUSION-IN-GEL PRECIPITIN TESTS BETWEEN GROUPS OF SERIAL GUINEA PIG SERA AND VARIOUS ORGAN EXTRACTS OF ADULT GUINEA PIG #4 WHO CO-HABITATED WITH AN INOCULATED GUINEA PIG, DEVELOPED A SIGNIFICANT HI TITER AGAINST DA VIRUS AND WAS SACRIFICED TWO WEEKS AFTER COMMENCEMENT OF CO-HABITATION:

serum changes observed	serum sets with greater than 4X HI rise		serum sets without greater than 4X HI rise	
	#	%	#	%
negative pre to positive post	47	35	4	12
positive pre to negative post	0	0	7	21
negative pre to negative post	39	28	15	44
positive pre to positive post	50	37	8	23
Total	136	100	34	100

Table 6

SUMMARY OF DIFFUSION-IN-GEL PRECIPITIN TESTS BETWEEN GROUPS OF SERIAL GUINEA PIG SERA AND VARIOUS ORGAN EXTRACTS OF BABY GUINEA PIG #8 WHO WAS SACRIFICED TWO WEEKS AFTER INTRA-NASAL AND INTRA-PERITONEAL INOCULATION OF LIVE DA VIRUS. GUINEA PIG #8 DEVELOPED A SIGNIFICANT HI TITER.

serum changes observed	serum sets with greater than 4X HI rise		serum sets without greater than 4X HI rise	
	#	%	#	%
negative pre to positive post	33	28	2	7
positive pre to negative post	2	1	1	3
negative pre to negative post	65	54	13	43
positive pre to positive post	20	17	14	47
Total	120	100	30	100

Table 7

SUMMARY OF DIFFUSION-IN-GEL PRECIPITIN TESTS BETWEEN GROUPS OF SERIAL GUINEA PIG SERA AND ORGAN EXTRACTS FROM ALL THE GUINEA PIGS EXPOSED TO THE DA VIRUS IN VIVO (i.e. GUINEA PIGS #2, 4, 8 and 34):

serum changes observed	serum sets with greater than 4X HI rise		serum sets without greater than 4X HI rise		all serum sets regardless of HI rise	
	#	%	#	%	#	%
negative pre to positive post	103	25	8	8	111	21
positive pre to negative post	2	0	9	9	11	2
negative pre to negative post	238	57	65	62	303	59
positive pre to positive post	73	18	22	21	95	18
Total	416	100	104	100	520	100

Table 8

SUMMARY OF DIFFUSION-IN-GEL PRECIPITIN TESTS BETWEEN GROUPS OF SERIAL GUINEA PIG SERA AND ORGAN EXTRACTS OF ADULT GUINEA PIG #27 WHOSE ORGANS WERE SUSPENDED IN SALINE AND INCUBATED WITH LIVE DA VIRUS AND APPROPRIATE CONTROLS IN VITRO:

serum changes observed	all serum sets vs. organs incubated with live DA virus		all serum sets vs. organs incubated with saline control	
	#	%	#	%
negative pre to positive post	0	0	0	0
positive pre to negative post	0	0	0	0
negative pre to negative post	90	100	90	100
positive pre to positive post	0	0	0	0
Total	90	100	90	100
paired pres both negative	18	100	18	100
paired pres both positive	0	0	0	0
paired pres which vary	0	0	0	0
paired pres without variance	18	100	18	100
Total	18	100	18	100

Table 9

SUMMARY OF DIFFUSION-IN-GEL PRECIPITIN TESTS BETWEEN GROUPS OF SERIAL GUINEA PIG SERA FROM ANIMALS DIRECTLY INOCULATED WITH LIVE DA VIRUS AND ORGAN EXTRACTS FROM GUINEA PIGS #2, 4, 5, 8 and 34:

serum changes observed	serum sets from inoculated animals with greater than 4X HI rise		serum sets from inoculated animals without greater than 4X HI rise	
	#	%	#	%
negative pre to positive post	127	37	0	0
positive pre to negative post	1	0	22	32
negative pre to negative post	184	53	36	52
positive pre to positive post	33	10	11	16
Total	345	100	69	100

Table 10

SUMMARY OF DIFFUSION-IN-GEL PRECIPITIN TESTS BETWEEN GROUPS OF SERIAL GUINEA PIG SERA FROM ANIMALS CO-HABITATING WITH ANIMALS INOCULATED WITH LIVE DA VIRUS AND ORGAN EXTRACTS FROM GUINEA PIGS #2, 4, 5, 8, and 34:

serum changes observed	serum sets from co-habitating animals with greater than 4X HI rise; i.e. infected		serum sets from co-habitating animals without greater than 4X HI rise; i.e. uninfected	
	#	%	#	%
negative pre to positive post	34	17	15	22
positive pre to negative post	3	1	0	0
negative pre to positive post	132	64	43	62
positive pre to positive post	38	18	11	16
Total	207	100	69	100

Table 11

SUMMARY OF DIFFUSION-IN-GEL PRECIPITIN TESTS BETWEEN GROUPS OF SERIAL GUINEA PIG SERA (DRAWN BEFORE EXPERIMENTAL VIRAL EXPOSURE) AND ORGAN EXTRACTS FROM ALL THE GUINEA PIGS EXPOSED TO THE DA VIRUS IN VIVO (i.e. GUINEA PIGS #2, 4, 8, and 34):

serum changes observed	serum sets with greater than 4X HI rise		serum sets without greater than 4X HI rise	
	#	%	#	%
paired pres both negative	85	82	0	0
paired pres both positive	3	3	0	0
paired pres which varied	16	15	0	0
paired pres without variance	88	85	0	0
Total	104	100	0	0

Table 12

SUMMARY OF DIFFUSION-IN-GEL PRECIPITIN TESTS BETWEEN GROUPS OF SERIAL GUINEA PIG SERA AND VARIOUS ORGAN EXTRACTS OF BABY GUINEA PIG #5 WHO WAS SACRIFICED BEFORE INOCULATIONS WERE BEGUN:

serum changes observed	serum sets with greater than 4X HI rise		serum sets without 4X or greater HI rise	
	#	%	#	%
negative pre to positive post	44	32	7	21
positive pre to negative post	2	2	13	38
negative pre to negative post	80	59	14	41
positive pre to positive post	10	7	0	0
Total	136	100	34	100

Table 13

SUMMARY OF DIFFUSION-IN-GEL PRECIPITIN TESTS BETWEEN: GROUPS OF SERIAL ISOLOGOUS GUINEA PIG SERA AND ISOLOGOUS GUINEA PIG ORGAN EXTRACTS; BETWEEN GROUPS OF SERIAL AUTOLOGOUS GUINEA PIG SERA AND AUTOLOGOUS GUINEA PIG ORGAN EXTRACTS AND BETWEEN ALL SERUM GROUPS AND ALL ORGAN EXTRACTS.

serum changes observed	isologous systems		autologous systems		all systems	
	#	%	#	%	#	%
negative pre to positive post	140	22	22	51	162	24
positive pre to negative post	26	4	0	0	26	4
negative pre to negative post	377	58	20	47	388	55
positive pre to positive post	104	16	1	2	105	15
Total	647	100	43	100	690	100

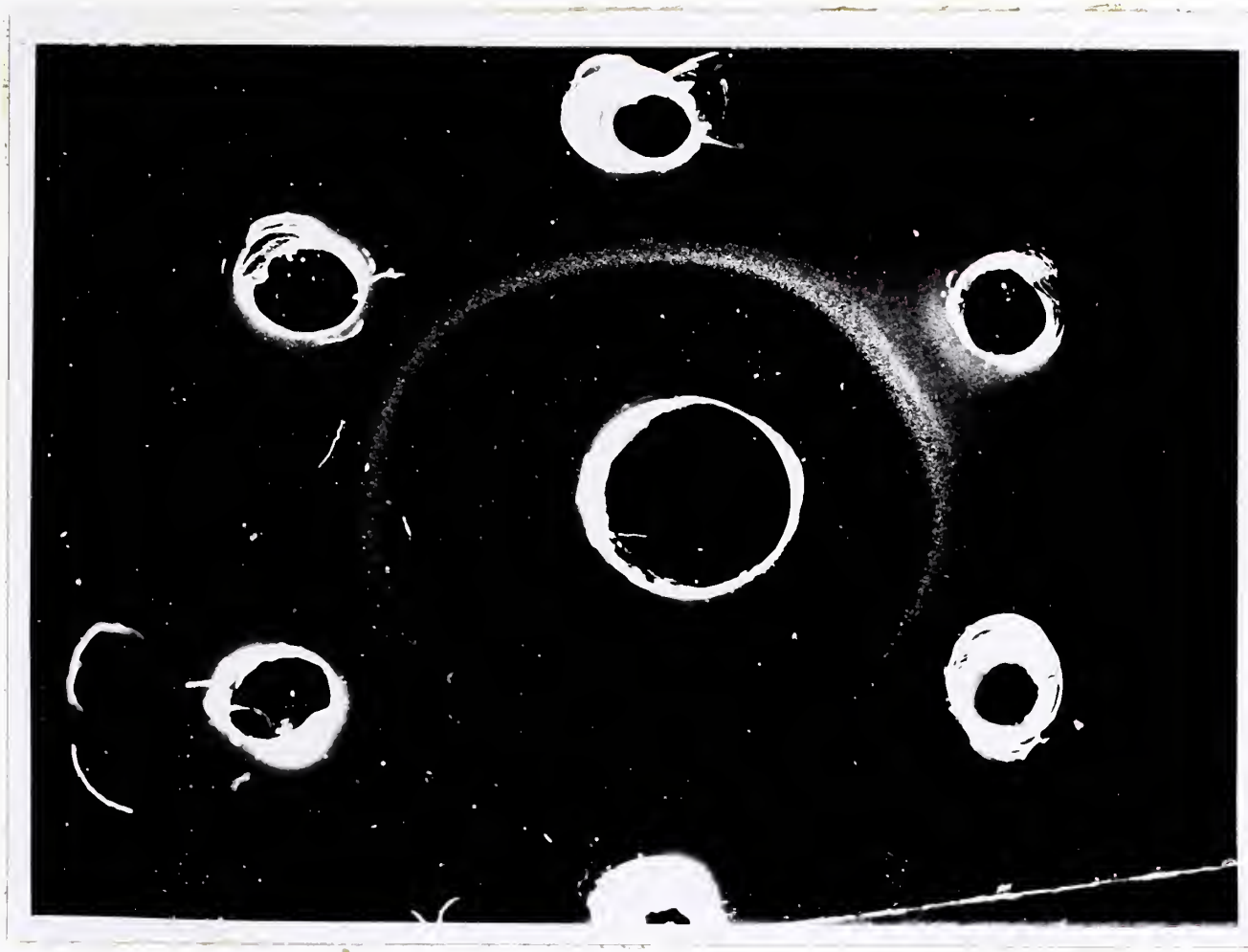

FIGURE 1: Photograph of dark field illuminated micro-Ouchterlony diffusion-in-gel precipitin system magnified many times. The six peripheral wells were filled with extracts from six different organs of the same guinea pig and the central well was filled with serum obtained from another guinea pig after exposure to the DA virus. The ring precipitin described in the text is seen, as well as two additional precipitin bands between the serum and the organ extract at 2 o'clock.

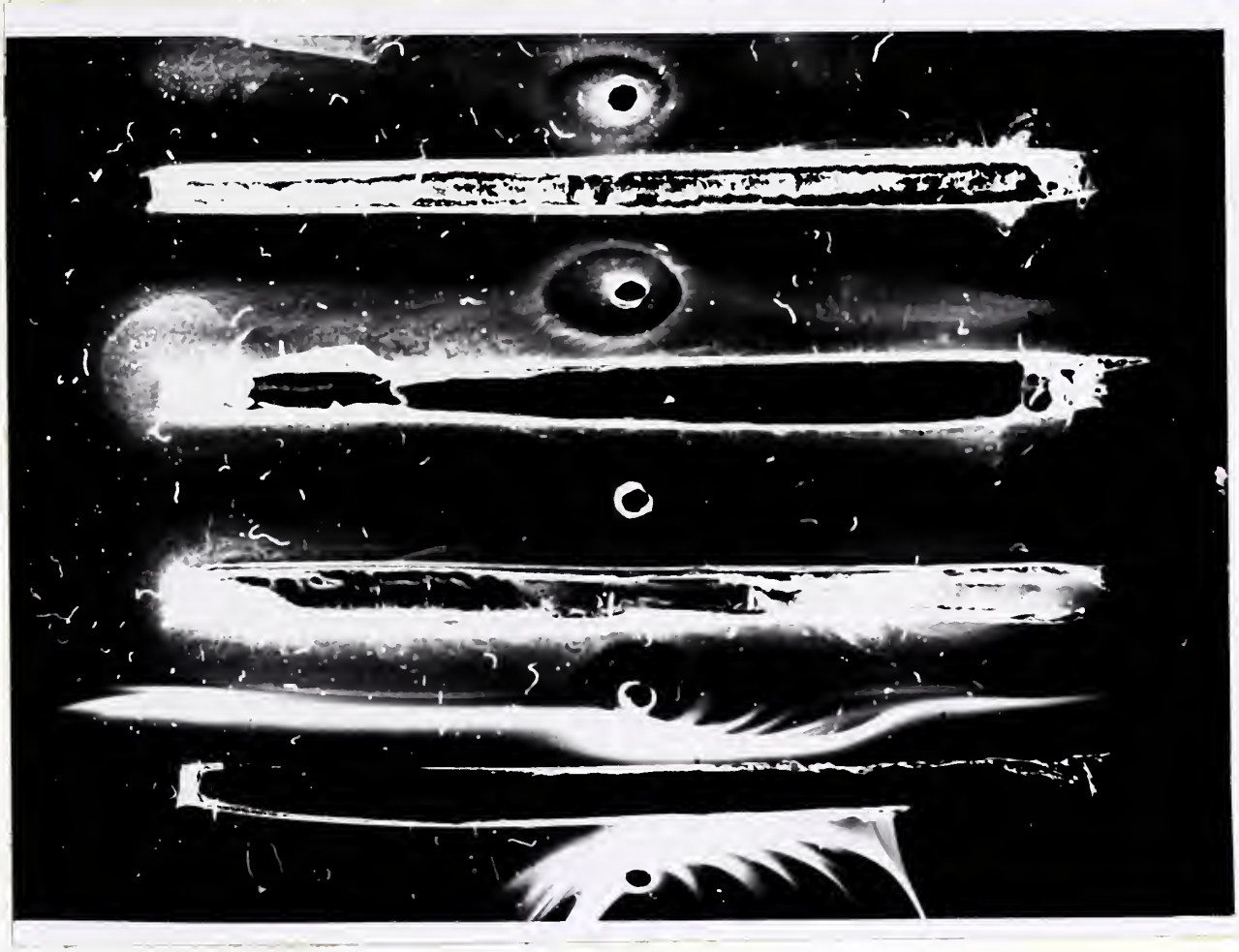

FIGURE 2: Photograph of dark field illuminated immunoelectrophoresis system with the positive electrode at the left. The top four circular wells were filled with sera from DA exposed guinea pigs and the bottom circular well with commercial pooled guinea pig serum. The top three rectangular troughs were filled with different organ extracts from a DA exposed guinea pig and the bottom trough with the commercial anti-guinea-pig-globulin made in a rabbit.

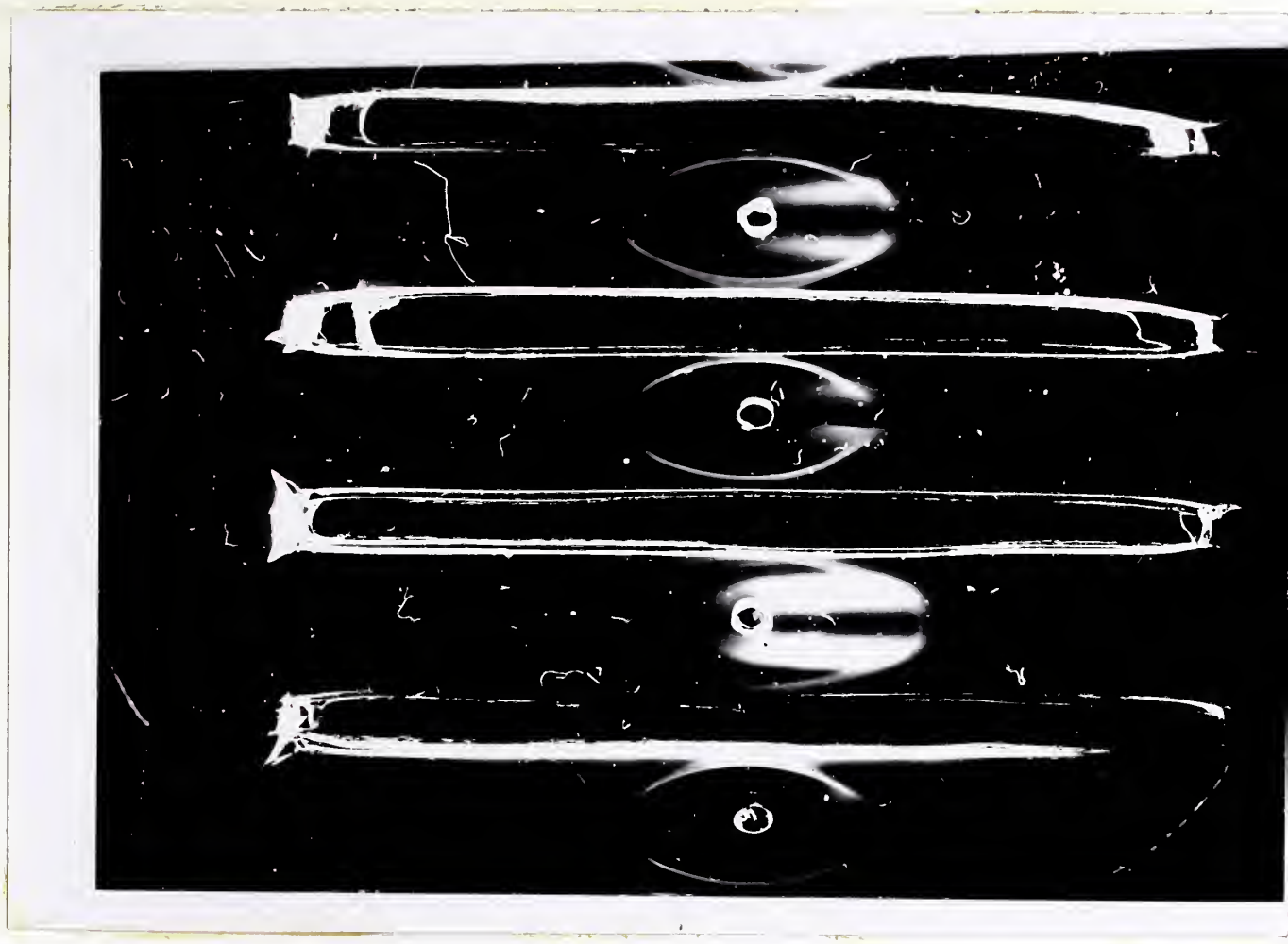

FIGURE 3: Photograph of dark field illuminated immunoelectrophoresis system with the positive electrode at the left. The circular wells were filled with organ extracts from a guinea pig exposed to DA virus and the troughs were filled with commercial anti-guinea-pig-globulin made in a rabbit.

BIBLIOGRAPHY

1. Florey, Sir Howard. "General Pathology". W. B. Saunders Co., Philadelphia, 1962.

2. Wissler, Robert W. Effect of Specific AB on Tissue Cells. Ann. Rev. Microbiol. 16: 265 (1962).

3. Gear, James. Tissue Special Antibodies; Discussion. In "Recent Progress in Microbiology". Charles C. Thomas, Springfield, Ill. 1959.

4. Gajdusek, D. Carleton. An "Autoimmune" Reaction Against Human Tissue Antigens in Certain Acute and Chronic Diseases. I. Serological Investigations. Arch. Int. Med. 101: 9 (1958).

5. Gardner, Edward, J. H. Wallace, M. C. Dodd, and C-S Wright. Antigenically Modified Red Cells in Chickens Infected with Newcastle Disease. Proc. Soc. Exptl. Biol. Med. 87: 253 (1954).

6. Kistner, Sten A. and Mario Stefanini. Studies on Platelets; XVII. An Experimental Study of the Development of Platelet Antibodies. J. Lab. and Clin. Med. 48: 846 (1956).

7. Briody, Bernard A. Action of Viruses on Red Blood Cells. Transactions of the New York Academy of Science II, 14: 231 (1952).

8. Moolten, S.E. and E. Clark. The RBC as a Vehicle of Virus Transport. I. Isolation and Identification of Blood Borne Viruses. Transactions of the New York Academy of Science II, 14: 232 (1952).

9. Moolten, S.E. and E. Clark. The RBC as a Vehicle of Virus Transport. II. Role of Blood Borne Viruses in Auto Hemagglutination and in Hemolytic Anemia. Transactions of the New York Academy of Science II, 14: 235 (1952).

10. Isacson, Peter, Robert W. McCollum and G. D. Hsiung. Studies of a Myxovirus Isolated from Human Blood. II. Sensitization of Erythrocytes. J. Immunol. 88: 291 (1962).

11. Burnet, F.M. and S. G. Anderson. The "T" AG of GP and Human Red Cells. Australia Jour. Exp. Biol. and Med. Sci. 25: 213 (1947).

12. Doniach, D. and I. M. Roitt. Auto-Antibodies in Disease. Ann. Rev. Med. 13: 213 (1962).

13. Mackay, Ian R. and D.C. Gajdusek. An "Autoimmune" Reaction Against Human Tissue Antigens in Certain Acute and Chronic Diseases. II. Clinical Correlations. Arch. Int. Med. 101: 30 (1958).

14. Raffel, Sidney. Immunologic Disease. Pediatrics 21: 849 (1958).

15. Steiner, Jan W. and Robert Volpe. Autoimmunization - a Possible Mechanism of Tissue Injury; I. Theoretical and Experimental Aspects. Canadian Medical Association Journal 84: 1165 (1961).

16. Waksman, Byron. Auto-immunization and the Lesions of Auto-immunity. Medicine 41: 93 (1962).

17. Dameshek, William, R. Schwartz and H. Oliner. Current Concepts of Auto-immunization: An Interpretive Review. Blood, The Journal of Hematology 17: 775 (1961).

18. Hotchin, John. The Biology of Lymphocytic Choriomeningitis Infection: Virus-Induced Immune Disease. Cold Spring Harbor Symp. Quant. Biol. 27: 479 (1962).

19. Marcus, Philip I. Dynamics of Surface Modification in Myxovirus-Infected Cells. Cold Spring Harbor Symp. Quant. Biol. 27: 351 (1962).

20. Hotchin, John E., S. M. Cohen, H. Ruska, and C. Ruska. Electron Microscopical Aspects of Hemadsorbtion in Tissue Cultures Infected with Influenza Virus. Virology 6: 689 (1958).

21. Chang, Pei. Wen. Studies on the Pathogenesis of Parainfluenzae Type 5 Infection and Its Implications. Thesis, Dept. of Epidem. and Pub. Health, Yale Univ., 1965.

22. Burnet, F. M. and S. G. Anderson. Modification of Human Red Cells by Virus Action. II. The Receptor Gradient for Virus Action in Human Red Cells. Brit. J. Exp. Path. 27: 228 (1946).

23. Hirst, G.K. Adsorbtion of Influenza Hemagglutinins and Virus by Red Cells. J. Exp. Med. 76: 195 (1943).

24. Kabat, Elvin A. "Blood Group Substances" Academic Press, N.Y. 1956, p. 26

25. Isacson, Peter, Robert W. McCollum and G.D. Hsiung. Studies of a Myxovirus Isolated from Human Blood. III. Serologic Reactions in Persons with Viral Hepatitis. J. Immunol. 88: 300 (1962).

26. Evans, Alfred S. and E. C. Curnen. Serological Studies on Infectious Mononucleosis and Other Conditions with Human Erythrocytes Modified by Newcastle Disease Virus. J. Immunol. 58: 323 (1948).

27. Burnet, F.M. and S. G. Anderson. Modification of Human Red Cells by Virus Action. II. Agglutination of Modified Human Red Cells by Sera from Cases of Infectious Mononucleosis. Brit. J. Exp. Path. 27: 236 (1946)

28. Evans, Alfred S. Serological Studies on Infectious Mononucleosis and Viral Hepatitis with Human Erythrocytes Modified by Different Strains of Newcastle Disease Virus. J. Immunol. 64: 411 (1950).

29. Moolten, S.E. and E. Clark. Viremia in Acute Hemolytic Anemia and in Autohemagglutination. Arch. Int. Med. 89: 270 (1952).

30. Morgan, Herbert. Acquired Hemolytic Anemia and Viremia. J. Lab. & Clin. Med. 40: 924 (1952).

31. Eyquen, A. and J. Dausset. Recherche des Proprietes Inhibrices de l'Agglutination par le Virus de la Maladie de Newcastle dan les Serum de Malades Attients d'Anemia Hemolotique Acquise et d'Autres Syndrome Hemolitiques. Ann. Inst. Pasteur 83: 407 (1952).

32. Workman, J.B., A. B. Snider and T. G. Woodland. Viremia in Acquired Hemolytic Anemia and Related Reticulo-Endothelial Diseases. Am. J. Med. 16: 612 (1954).

33. De Reuck, A. V. S. and Julie Knight (Eds) "Ciba Foundation Symposium on Cellular Injury" Little Brown, Boston, 1964.

34. Hoyle, L., R. W. Horne and A. D. Waterson. The Structure and Composition of the Myxoviruses. II. Components Released from the Influenza Virus Particle by Ether. Virology 13: 448 (1961).

35. Cates, M., A. C. Allison, D. A. J. Tyren and A. T. James. Origin of Lipids in Influenza Virus. Cold Spring Harbor Symp. Quant. Biol. 27: 293 (1962).

36. Burnet, F. M. "The Clonal Selection Theory of Acquired Immunity" Cambridge University Press, London, 1959.

37. Knight, C. A. Precipitin Reactions of Highly Purified Influenza Viruses and Related Materials. J. Exp. Med. 83: 281 (1946).

38. Holden, David Morgan. The Blood Group Activity of Myxoviruses. Thesis, Yale University School of Medicine, 1963.

39. Morgan, Council, Konrad C. Hsu, Richard A. Rifkind, Alice W. Knox and Harry M. Rose. The Application of Ferritin-Conjugated Antibody to Electron Microscopic Studies of Influenza Virus in Infected Cells. J. Exp. Med. 114: 825 (1961).

40. Roane, Philip R. and B. Roizman. Studies of the Determinant Antigens of Viable Cells. II. Demonstration of Altered Antigenic Reactivity of Hep-2 Cells Infected with Herpes Simplex Virus. Virology 22: 1 (1964).

41. Habel, Karl. Antigenic Properties of Cells Transformed by Polyoma Virus. Cold Spring Harbor Symp. Quant. Biol. 27: 433 (1962).

42. Hackett, E. and M. Beech. Autoimmune Complement-Fixation Reaction in 1,014 Patients. Brit. J. Med. 2: 17 (1960).

43. Atta, Mohamad and Bengt Sievert. Thrombocytopenia Purpura After Morbilli. Acta Pediatrica 42: 364 (1953).

44. Alford, Charles A., F. A. Neva and T. H. Weller. Virologic and Serologic Studies on Human Products of Conception After Maternal Rubella. N.E. Jour. Med. 271: 1275 (1964).

45. Hsiung, G.D. The Use of Agar Overlay Cultures for Detection of New Virus Isolates. Virology 9: 717 (1959).

46. Hsiung, G.D., Peter Isacson and Robert W. McCollum. Studies of a Myxovirus Isolated from Human Blood. I. Isolation and Properties. J. Immunol. 88: 284 (1962).

47. Hsiung, G.D., Peter Isacson and Robert W. McCollum. Studies of a Myxovirus Isolated from Human Blood. I. Isolation and Characterization. J. Immunol. 88: 284 (1962).

48. Hsiung, G.D., P. W. Chang, P. P. Cuadrado and P. Isacson. Studies of Parainfluenza Viruses. III. Antibody Responses of Different Animal Species Following Immunization. J. Immunol. (1964) in press.

49. Melnick, Joseph L. Tissue Culture Techniques and Their Application to Original Isolation, Growth and Assay of Poliomyelitis and Orphan Viruses. Ann. N.Y. Acad. Sci 61: 754 (1955).

50. Hsiung, G.D. Use of Human Kidney Cultures in the Study of Enteroviruses. Proc. Soc. Exp. Biol. Med. 102: 612 (1959).

51. "Diagnostic Procedures for Virus and Rickettsial Diseases" American Public Health Association, 1956, New York.

52. Hsiung, G.D. "Diagnostic Virology" Yale Univ. Press, New Haven, 1964.

53. Dreschen, J., A. V. Hennessy and F. M. Davenport. Photometric Methods for the Measurement of Hemagglutinating Viruses and Antibody. J. Immunol. 89: 794 (1962).

54. Mizutani, H. $BaSO_4$ Purification of Virus. Nature 198: 109 (1963).

55. Broberger, Ove and Peter Perlmann. Autoantibodies in Human Ulcerative Colitis. J. Exp. Med. 110: 657 (1959).

56. Ouchterlony, Orjan. Diffusion-in-Gel Methods for Immunological Analysis. Progress in Allergy 5: 1 (1958).

57. Crowle, A. J. "Immunodiffusion" Academic Press, N.Y. 1961.

58. Crowles, Alfred J. Interpretation of Immunodiffusion Tests. Ann. Rev. Microbiol. 14: 161 (1960).

59. Ouchterlony, O. Gel Diffusion Techniques. In "Immunological Methods" J. F. Ackeroyd, Editor. Blackwell's, Oxford 1964, p. 55.

60. Korngold, L. and G. Van Leeuwen. The Effect of the Antigen's Molecular Weights on the Curvature of the Precipitin Line in the Ouchterlony Technic. J. Immunol. 78: 172 (1957).

61. Rosen, Fred S. The Macroglobulins. New England J. Med. 267: 491 (1962).

62. Marrach, J. R. Sensitivity and Specificity of Methods of Detecting Antibodies. Brit. Med. Bull. 19: 178 (1963).

63. Feinberg, Joseph C. A New Quantitative Method for Antigen Antibody Titrations in Gels. Nature 177: 530 (1956).

64. Wright, S. T. C. A Quantitative Serum-Agar Technique. Nature 183: 1282 (1959).

65. Ackroyd, J. F. "Immunological Methods" Blackwell's , 1964.

66. Roitt, I. M., D. Doniach, P. N. Campbell and R. V. Hudson. Auto-Antibodies in Hashimoto's Disease (Lymphadenoid Goiter). Lancet 271; II: 820 (1956).

67. Goudie, R. B., J. R. Anderson, K. G. Gray, D. H. Clark, I. P. C. Murry and G. P. McNicol. Precipitin Tests in Thyroid Disease. Lancet 273 II: 976 (1957).

68. Roitt, I. M. and D. Doniach. Auto-Immunization in Thyroid Diseases. Proc. Royal Soc. Med. 50: 958 (1957).

69. Liu, Charlotte T. and Wallace W. McCrory. Autoantibodies in Human Glomerulonephritis and Nephrotic Syndrome. J. Immunol. 81: 492 (1958).

70. Deicher, Helmuth R., R. H. Halsted, H. G. Kunkel. Anti-Cytoplasmic Factors in the Sera of Patients with SLE and Certain Other Diseases. Arthritis and Rhematism 3: 1 (1960).

71. Kidd, John G. and W. F. Friedewald. A Natural Antibody that Reacts in vitro with a Sedmientable Constituent of Normal Tissue Cells. J. Exp. Med. 76: 557 (1942).

72. Clouoh, Paul W. Autoimmunization and Autoantibodies. Ann. Int. Med. 52: 930 (1960).

73. Witebsky, E., N. R. Rose, K. Terplan, John R. Paine and R. W. Egan. Chronic Thyroiditis and Autoimmunization. JAMA 164: 1439 (1959).

74. Waksman, Byron. Experimental Allergic Encephalomyelitis and the "Auto-Allergic" Diseases. Int. Arch. Allergy and Appl. Immunol. 14: suppl. 1-87 (1959).

75. Trotter, W. R., G. Belyavin and A. Waddams. Precipitating and Complement-Fixing Antibodies in Hashimoto's Disease. Proc. Royal Soc. Med. 50: 961 (1957).

76. Kantor, F. S. and R. M. Cole. A Fibrinogen Precipitating Factor of Group A Streptococci. Proc. Soc. Exptl. Biol. Med. 102: 146 (1959).

77. Ho, Monti. Inteferons. N. E. Jour. Med. 266: 1258 and 1313 (1962).

78. Waksman, Byron. The Toxic Effects of the Antigen Antibody Reaction on the Cells of the Hypersensitive Reactor. In "Cellular and Humoral Aspects of the Hypersensitive States" H. Sherwood Lawrence, Editor., Hoeber & Harper, N.Y. 1959.

79. Grabar, Pierre. Auto Antigenicity. "Recent Progr. in Microbiology" Charles C. Thomas, Springfield, Ill. 1959.

80. Milgrom, F. Tissue Specific Antibodies, Discussion. In "Recent Progress in Microbiology" Charles C. Thomas, Springfield, Ill. 1959.

81. Muschel, Louis H., L. A. Simonton, P. A. Wells, and E. H. Fife. Occurrence of Complement-Fixing Antibodies Reactive with Normal Tissue Constituents in Normal and Disease States. J. Clin. Invest. 40: 517 (1961).

82. Battisto, Jack R. Natural Occurrence of Delayed Type Iso-Hypersensitiveness. Nature 187: 69 (1960).

83. Grabar, Pierre. The Problem of Auto-Antibodies. Texas Reports on Biology and Medicine 15: 1 (1957).

84. Jensen, Keith and Thomas Fronsis. Antigen-Antibody Precipitates in Solid Medium with Influenza Virus. J. Immunol. 70: 321 (1953).

85. Brown, F. and Joan Crick. Specific Precipitin Reactions with the Viruses of Foot-and-Mouth Disease and Vesicular Stomatitis. Nature 179: 316 (1957).

86. Smith, Wilson, G. Belyavin and F. W. Sheffield. The Host-Tissue Component of Influenza Viruses. Proc. Royal Soc. 143: 504 (1955).

87. Martin, C. M. and J. H. Jandl. Inhibition of Virus Multiplication by Transferrin. J. Clin. Invest. 38: 1024 (1960).

YALE MEDICAL LIBRARY

Manuscript Theses

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Yale Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by has been used by the following persons, whose signatures attest their acceptance of the above restrictions.

NAME AND ADDRESS	DATE
Michael Canty 182 South St.	April 29, 1981